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SYNERGISM IN THE KINETIC REACTIONS OF O_2 AND CO_2 WITH HUMAN RED BLOOD CELLS*

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SYNERGISM: *Physiol.* 'Co-operative action of discrete agencies such that the total effect is greater than the sum of the two effects taken independently' (Webster, 1961).

When arterial blood enters a tissue capillary it exchanges oxygen and carbon dioxide with its environment. O_2 at a higher partial pressure in the plasma diffuses into the tissues. The equilibrium of the reaction,

$$O_2 + Hb \underset{k_c}{\overset{k'_c}{\rightleftharpoons}} O_2Hb$$

is disturbed and the dissociation reaction controlled by the rate constant k_c proceeds until a new equilibrium is reached or until the blood leaves the capillary and enters the vein. In a similar manner CO_2 at a higher partial pressure in the tissues diffuses into the blood where some of it remains in physical solution, some reacts with certain amine groups of haemoglobin to form carbamino compounds and some reacts with water in the presence of erythrocyte carbonic anhydrase to form carbonic acid. Even in the absence of the enzyme the hydration reaction occurs, though at a much slower rate.

These reactions of O_2 and CO_2 in blood are not independent. It has long been known (Bohr, Hasselbalch & Krogh, 1904) that CO_2 added to blood decreases the amount of O_2 that is held in association with Hb at a given P_{O_2}, that is, CO_2 shifts the O_2Hb-equilibrium curve to the right (Bohr shift). Christiansen, Douglas & Haldane (1914) showed that oxygenation of haemoglobin promotes the dissociation of CO_2 from blood because O_2Hb is a stronger acid than Hb (Haldane effect).

In the tissue capillaries the Bohr shift and Haldane effect are mutually beneficial in that at equilibrium more O_2 is delivered and more CO_2

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removed by the blood than would occur by either reaction alone. However, the results are additive and predictable from known steady-state data. We do not call this type of interaction synergism. But if we consider the transient phases of these reactions, we can ask the questions: does the addition of CO_2 to blood in any way affect the *rate* at which O_2Hb dissociates in the tissue capillaries and, conversely, does the removal of O_2Hb in any way affect the *rate* at which CO_2 is taken up in the blood?

Search of the literature reveals no evidence or consideration of possible synergism between the kinetic reactions of O_2 and CO_2 with haemoglobin although the reaction rates of the individual components have been measured. Roughton and co-workers have made extensive measurements of the reactions of O_2 with haemoglobin in intact red cells and in solution (Gibson, 1959; Roughton, 1959). As early as 1923 Hartridge & Roughton showed that lowering the pH increased the rate of dissociation of O_2Hb in dilute solution. Recently Lawson (1962) showed the same effect in human red cells. At 22° C the rate of O_2Hb dissociation was approximately doubled at pH 7·2 compared to pH 7·8. But these rates were measured using haemoglobin solutions and intact cells pre-incubated at various pHs. There was no change in pH or P_{CO_2} at the time of mixing in the rapid reaction apparatus. Barcroft (1914) showed that acidifying the blood speeded the rate at which whole blood was reduced (deoxygenated) when nitrogen was bubbled through it. The results, however, are only of qualitative value since the experiment consisted of bubbling nitrogen through a large flask of blood and following the deoxygenation rate over a period of many minutes, which is hundreds of times longer than the time available for exchange in the capillaries.

Dirken & Mook (1931) studied the rate of CO_2 entry into erythrocytes by examining ultrafiltrates of the extracellular fluid in their rapid reaction apparatus at various times after mixing. They followed the chloride shift and found a half time (t_{50}) of 0·39 sec at 15° C. More recently Roughton & Rupp (1958) re-studied the chloride shift and found t_{50} = 0·80 sec normally. At the same time they computed that the carbamino reaction would be virtually complete in 0·10 sec. There are no data in the foregoing on the Bohr shift reaction, but only on the CO_2 hydration and carbamino reactions that provide the basis for it. Craw, Constantine, Morello & Forster (1963) studied the actual kinetics of the Bohr shift in dilute human red cell suspensions. They found the Bohr shift to be relatively slow (t_{50} = 0·12 sec) compared to the deoxygenation rate of O_2Hb (t_{50} = 0·012 sec). The carbonic anhydrase inhibitor acetazolamide markedly impeded the reaction. They concluded that because of its slowness the Bohr shift reaction must be partially rate limiting in O_2 exchanges in tissue capillaries.

Other studies of the interactions of O_2 and CO_2 with blood and in

pulmonary gas exchange have recently been made by Visser (1960), Defares & Visser (1962) and Bernard (1960, 1961). These analyses are all based on the steady-state Bohr shift and Haldane effect. They do not give much insight into the transients that exist normally. Visser (1960), however, shows clearly that CO_2 exchanges cannot really proceed much faster than O_2 exchanges because of the known interactions.

In this work we have experimentally determined the rate of dissociation of O_2Hb in dilute human red cell suspensions at 37° C, then the same reaction in the presence of a sudden increase in CO_2 and finally the rate of the Bohr shift reaction alone. We expected to find a two-stage dissociation of O_2Hb in the presence of increased CO_2 as implied by Craw and associates. Instead we found an accelerated one-stage reaction in which both O_2 and CO_2 reactions are faster. This is true synergism unpredicted from previous work on the reactions measured separately.

METHODS

We used the Hartridge–Roughton continuous flow principle in which rapidly reacting materials mix and flow through a narrow-bore tube; the progress of the reaction is measured at various distances and therefore at different times after mixing (Hartridge & Roughton, 1923). We used a modification of our rapid-reaction apparatus (Staub, Bishop & Forster, 1961). It consists of two precision-bore, motor-driven syringes from which fluids of known composition can be driven at precisely known volume flow rates. The reactants are mixed in a four-tangential-jet mixing chamber within 2–3 msec and the mixture then flows with turbulent motion down a uniform bore ($0{\cdot}158 \pm 0{\cdot}002$ cm diameter) stainless-steel observation tube along which six small oxygen electrodes (Staub, 1961) are located (Figs. 1 and 2). Once the reactants mix in the mixing chamber ($t = 0$) there is no further change in the total concentration of any of the materials. Since the reaction system is a closed one we can use the solubility of O_2 in the mixture and the measured changes in extracellular P_{O_2} to calculate the change in O_2Hb saturation at each time interval.

The outputs of the oxygen electrodes were amplified by differential electrometer amplifiers and recorded on three dual-channel strip chart recorders. The volume flow rates of each reactant were also recorded (Staub *et al.* 1961). The major improvement in design for this experiment from our previous work is the use of the six simultaneously recording oxygen electrodes at fixed positions along the observation tube. This arrangement has several advantages: (1) all electrodes can be checked and calibrated simultaneously; (2) an entire reaction curve can be recorded in a single experiment thereby minimizing the variations that may occur when six separate experiments are needed to obtain six different points on one curve; (3) complete reaction curves can be determined and checked as quickly as the syringes can be re-filled thereby reducing the time for experiments and possible long-term changes; (4) far less reactant materials are needed. However, using six electrodes requires careful preparation and checking. To reduce problems due to faulty membranes covering the electrodes we used 25μ thick 'Teflon' cast film (DuPont Corp., tetrafluoroethylene resin). The responses of the electrodes were slower than those previously reported but with large syringes we were able to continue flow up to 30 sec and to obtain good response plateaux. All apparatus and reactant fluids were kept at $37 \pm 0{\cdot}5$° C in water baths.

Plan of the experiments. We carried out five series of experiments designed to determine:

I, the rate of deoxygenation of human erythrocytes;

II, the rate of deoxygenation with simultaneous Bohr shift;

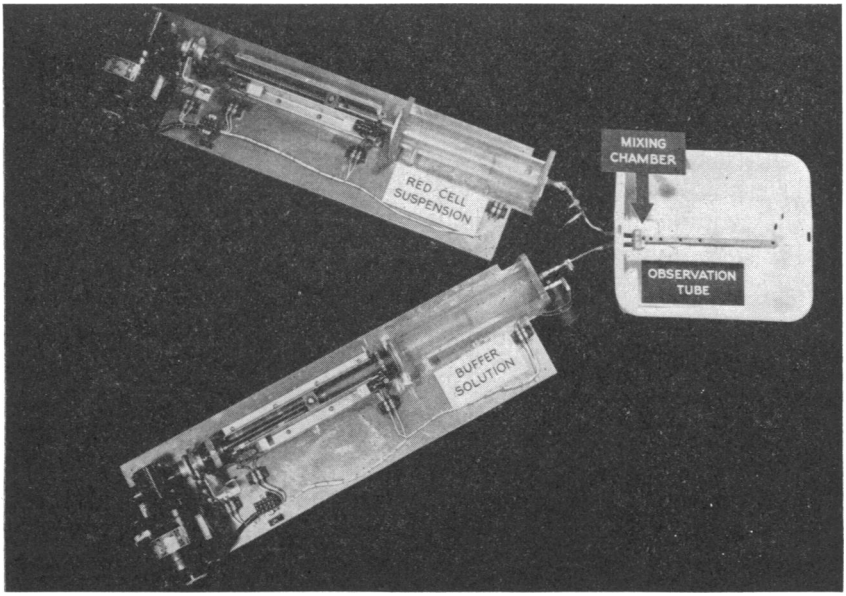

Fig. 1. Photograph of basic components of rapid reaction apparatus. The two motor-driven syringes, the mixing chamber and the six-port observation tube are shown.

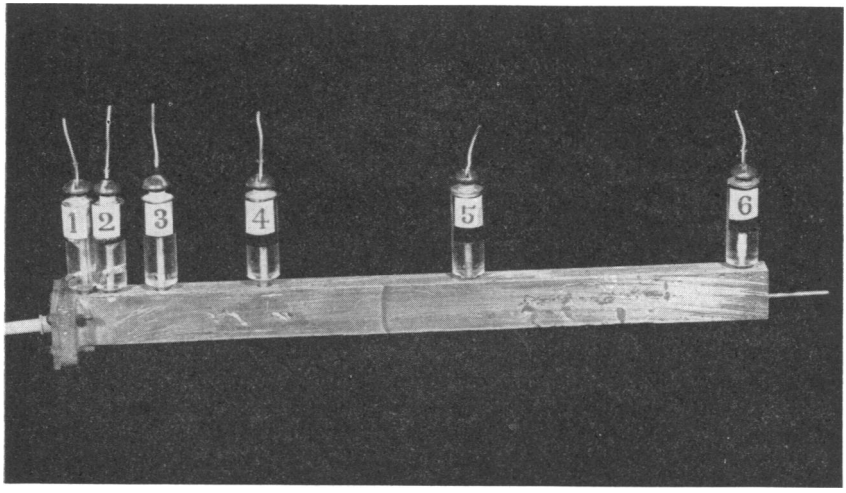

Fig. 2. Observation tube with six O_2 electrodes in place. The mixing chamber is at the left end of the tube. The stainless-steel tube in which the mix actually flows can be seen at the right projecting from the electrode holder.

III, the rate of deoxygenation with Bohr shift in the presence of the carbonic anhydrase inhibitor acetazolamide;

IV, the rate of the Bohr shift alone;

V, the rate of the Bohr shift in the presence of acetazolamide.

Series I–III require the same reactants and will be described together. For each experiment we prepared a red cell suspension containing approximately 150 ml. freshly drawn heparinized human venous blood diluted to 1500 ml. with a modified Gey and Gey buffer solution (Staub *et al.* 1961). The blood was agitated throughout by a small motor-driven stirrer and bubbled by pre-warmed and humidified gas containing 13·87 % O_2 and 5·63 % CO_2 (Scholander analysis) from large gas cylinders. All glass-ware and tubing in the blood reservoir were coated with Dow–Corning Antifoam A. In addition, a large stainless-steel sponge coated with antifoam was suspended over the blood to keep foam to a minimum. After 20 min of bubbling through a coarse fritted glass bubbler, the P_{O_2} and P_{CO_2} of the blood were in equilibrium with the gas phase and remained so for the duration of the experiment. Blood samples taken at regular intervals during the experiment showed less than 2 % variation in haemoglobin concentration and essentially no plasma haemoglobin. The pH of the buffer when equilibrated with 40 mm Hg P_{CO_2} was 7·40 ± 0·01 at 37° C.

Two reservoir bottles of Gey and Gey buffer solution were bubbled with gas at P_{O_2} = 2 mm Hg and P_{CO_2} = 40 and 100 mm Hg respectively. The pH of the low-CO_2 buffer was 7·40 ± 0·01 and of the high-CO_2 buffer was 7·03 ± 0·02, both at 37° C.

In series I the red cell suspension was mixed with the low-CO_2 buffer, thereby giving a change of P_{O_2} but no initial change of P_{CO_2} or pH. In series II the blood was mixed with the high-CO_2 buffer giving the same initial change in P_{O_2} and a sudden large increase in P_{CO_2}. In series III acetazolamide in quantities up to 5 mg/100 ml. was added to the blood reservoir. After incubation for 25–75 min, the experiment with blood and high-CO_2 buffer was run again. In every experiment the mixture ratio of red cell suspension to buffer was 1:2. Calculations had shown that this ratio would give a good shift in extracellular P_{O_2} and yet would not use up the syringe of buffer too rapidly. In these experiments the length of the observation tube was 28 cm and with the flow rates selected permitted recording of P_{O_2} at six points ranging from 5 to 160 msec after the beginning of the reaction. Each experiment was run 2–3 times in succession after suitable checking runs and calibrations to test all the equipment. At the conclusion another calibration run was used to be certain the electrodes had remained reliable. In practice the final controls were nearly always the same as the initial except when an electrode failed because of a leaky membrane. This event was obvious in the records.

Series IV and V required the same buffer solution and red cell suspension but a different gas supply. The P_{O_2} of the blood and buffer were made equal, and only the P_{CO_2} and pH in the reactants differed. The blood was equilibrated with 9·94 % O_2 and 13·54 % CO_2 (pH = 7·07 ± 0·02) and the buffer was equilibrated with 9·94 % O_2 and 0 % CO_2 (pH = 9·24 ± 0·02). In series IV at time zero the extracellular portion of the mixture had a P_{O_2} of approximately 70 mm Hg and was in equilibrium with the P_{O_2} in the cells. The initial P_{CO_2}, however, was about 95 mm Hg in the cells but only 32 mm Hg outside since the mixing proportions of blood to buffer were 1:2 as before. In series V acetazolamide in quantities up to 5 mg/100 ml. was added to the blood reservoir and the experiment repeated after 30 min. Since both the blood and buffer had the same P_{O_2} it was necessary to have a third syringe containing water at a known P_{O_2} in order to obtain an absolute calibration for the P_{O_2} changes during the reaction. The fluid for the third syringe was bubbled with gas containing 6·25 % O_2. This was chosen because it was below the theoretical limit of P_{O_2} change in the experiment, and with the aid of zero suppression and scale expansion we were able to make the interval 44 mm Hg to 70 mm Hg P_{O_2} cover about 5 in. of the 6 in. recorder paper width. We were thus able to read the P_{O_2} changes during the reaction to about 0·25 mm Hg.

In all the series of experiments we used the blood of five healthy males. The initial O_2Hb saturations were calculated from the known P_{O_2} and pH using the oxygen equilibrium curve of Bock, Field & Adair (1924). Although there may be small individual variations in the O_2Hb curves the error in computing the absolute value for the reaction constant, k_c, would only be 1–2 % at the most. Since we were mainly interested in relative changes in this experiment even this small error would cancel out because we used each subject as his own control.

In the Bohr shift series (IV and V), five oxygen electrodes were spread out in time from 15 to 500 msec with the sixth at 5000 msec. These changes were accomplished by decreasing the flow rates slightly and by extending the observation tube to 63 cm and then adding a large glass coil (volume = 12 ml.) before the final point.

RESULTS

The results are given in Table 1 (series I–III) and Table 2 (series IV and V). The initial rate constant, half time (t_{50}) and 90 % time (t_{90}) are given for each experiment (average of 2–3 runs) on each subject. Figures 3–6 are representative of the experimental curves.

TABLE 1. Important kinetic values for all the experiments on deoxygenation (I), deoxygenation plus Bohr shift (II) and deoxygenation plus Bohr shift after acetazolamide (III)

		I Deoxygenation			II Deoxygenation plus Bohr shift			III Deoxygenation plus Bohr shift and acetazolamide		
Subject	Expt. no.	k_c (sec^{-1})	t_{50} (msec)	t_{90} (msec)	k_c (sec^{-1})	t_{50} (msec)	t_{90} (msec)	k_c (sec^{-1})	t_{50} (msec)	t_{90} (msec)
N. C. S.	2	5·2	13	56	6·4	19	63	—	—	—
	4	5·7	14	58	6·8	17	69	—	—	—
	6	5·1	12	50	6·4	14	61	—	—	—
	10	4·9	12	52	5·7	17	61	—	—	—
	12	5·9	10	41	7·2	12	53	6·6	14	> 200
	14	6·3	12	41	8·0	14	54	6·3	20	> 200
T. N.	1	5·0	14	59	5·8	18	67	—	—	—
	3	5·4	13	55	6·4	16	60	—	—	—
	5	3·9	13	63	5·0	18	79	—	—	—
	9	4·2	16	57	5·3	21	65	4·5	25	> 200
	13	4·4	10	31	5·9	14	66	4·2	18	> 200
H. N.	8	5·0	13	51	5·9	12	56	—	—	—
	11	3·8	16	59	5·2	18	54	4·1	29	> 200
T. V.	7	7·7	10	60	11·5	10	72	—	—	—
Averages	—	5·2	13	53	6·5	16	62	5·2	21	> 200

The data on deoxygenation (series I) from an initial saturation of 97 % are given in the first section of Table 1 for 14 experiments on four different subjects. Figure 3 shows the plotted points for three successive runs on one subject. The scatter of points was so small that in most of our experiments only two runs were done. Smooth curves were drawn through the points from the origin. In Fig. 3 the reaction is essentially complete at

0·150 sec. The theoretical final equilibrium point agrees almost exactly with the determined value.

The initial over-all cellular deoxygenation rate constant k_c is a measure of the rate of reaction at $t = 0$. As pointed out by Roughton (1959) it is not a true chemical rate constant because it includes the diffusion process of O_2 out of the red cell. However, given the same initial saturation as in each of our experiments it provides quantitative data on the physiological kinetics of the dissociation of O_2 from O_2Hb in red cells. The equation to be used to calculate k_c is

$$\left(\frac{d(O_2Hb)}{dt}\right)_{t=0} = k_c(O_2Hb),$$

where O_2Hb is the initial saturation and $d(O_2Hb)/dt$ is the slope of experimentally determined kinetic curve at zero time. In the example in Fig. 3, the initial slope is 375 %/sec. Inserting that value into the equation at an initial saturation of 97 % we obtain $k_c = 3{\cdot}9\ \text{sec}^{-1}$.

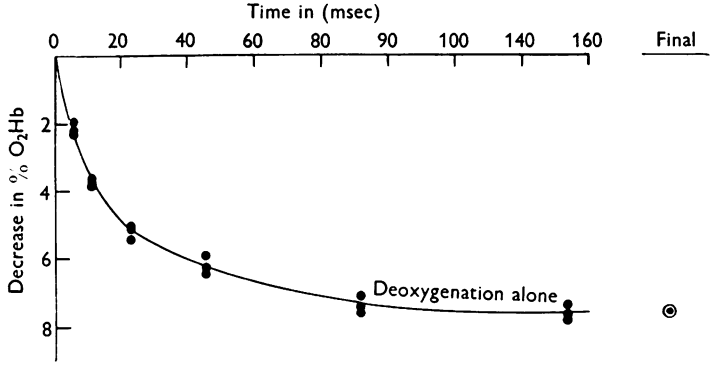

Fig. 3. Time course of deoxygenation of a human red blood cell suspension induced by a sudden decrease in P_{O_2}. Three separate runs and the final theoretical equilibrium point (⊙) are shown. Expt. 5. Subject, T. N.

The data on deoxygenation in the presence of a simultaneous Bohr shift (series II) are given in the second part of Table 1. Figure 4 shows the points on the time course for two successive runs both for deoxygenation alone and for deoxygenation plus the Bohr shift. All the points for the Bohr shift curve lie below the comparable points for the deoxygenation alone. The two curves are smooth. There is no suggestion of a two-step reaction. All experiments of this series behaved in the same fashion. It is very important to note that the completion of the combined reaction is only slightly slower than for deoxygenation alone. Since the over-all change for the Bohr shift plus deoxygenation reaction is the greater by about 4 % O_2Hb this means that molecule for molecule deoxygenation with the

simultaneous Bohr effect drives O_2 off haemoglobin faster than deoxygenation alone.

The data for the effect of the carbonic anhydrase inhibitor acetazolamide on the rate of deoxygenation plus Bohr shift (series III) are given in the third part of Table 1. The experiments labelled 13 and 14 form a subgroup of the set. The first three experiments were performed by adding single doses of acetazolamide to the blood reservoirs and waiting 25–30 min before running the experiment. The doses were approximately 5 mg/100 ml. of blood suspension. In terms of whole blood these concentrations were about 50 mg/100 ml., far more than is generally believed

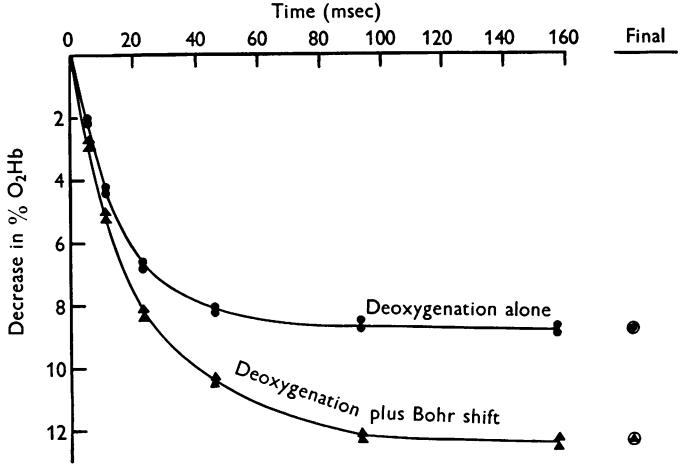

Fig. 4. Time course of deoxygenation alone and of deoxygenation plus Bohr shift. Two separate runs are shown on each curve. Expt. 6. Subject, N. C. S.

TABLE 2. Effect of graded concentrations of acetazolamide on the rate of deoxygenation + Bohr shift

Expt. no.	Comment	k_c (sec^{-1})
13	Deoxygenation alone	4·4
	Deoxygenation + Bohr shift	5·9
	Deoxygenation + Bohr shift + 0·4 mg/100 ml. acetazolamide	5·9
	Deoxygenation + Bohr shift + 0·9 mg/100 ml. acetazolamide	5·1
	Deoxygenation + Bohr shift + 2·9 mg/100 ml. acetazolamide	4·2
14	Deoxygenation alone	6·3
	Deoxygenation + Bohr shift	8·0
	Deoxygenation + Bohr shift + 0·5 mg/100 ml. acetazolamide	7·5
	Deoxygenation + Bohr shift + 1·9 mg/100 ml. acetazolamide	6·9
	Deoxygenation + Bohr shift + 3·9 mg/100 ml. acetazolamide	6·3

necessary to inhibit over 99% of circulating erythrocyte carbonic anhydrase. The experimental results, however, showed only partial (50–75%) blockage of the Bohr shift effect, indicating either incomplete inhibition of the enzyme or that part of the Bohr effect is due to something

besides the CO_2 hydration reaction. To determine which explanation was correct we performed Expts. 13 and 14 using graded doses of acetazolamide. The inhibitor was added in three successive doses over a $1\frac{1}{4}$ hr period. After each dose a waiting period of about 20 min was allowed before performing an experiment. Table 2 shows the trend of the initial rate constant in these two experiments. Although the total amount of acetazolamide added was less than in the first three experiments it was

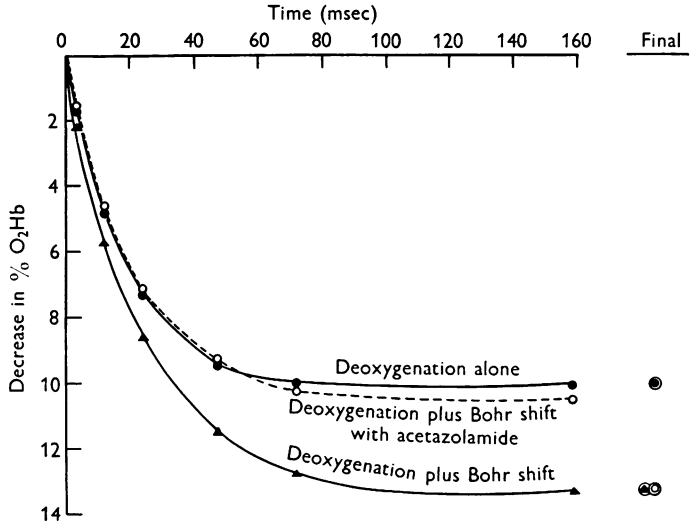

Fig. 5. Time course of deoxygenation alone, deoxygenation plus Bohr shift and deoxygenation plus acetazolamide-inhibited Bohr shift. Each point on each curve is the average of two successive runs. Final equilibrium points are shown. Note that the two Bohr shift curves eventually end up at the same point. Expt. 14. Subject, N. C. S.

TABLE 3. Important kinetic values for the experiments on the Bohr shift alone (IV) and the Bohr shift after acetazolamide (V)

		IV Bohr shift		V Bohr shift after acetazolamide	
Subject	Expt. no.	Initial rate (%/sec)	t_{50} (sec)	Initial rate (%/sec)	t_{50} (sec)
N. C. S.	15	80	0·150	19	$\geqslant$ 0·500
Y. S.	16	78	0·152	24	$\geqslant$ 0·500
H. N.	17	85	0·155	18	$\geqslant$ 0·500
Average		81	0·152	20	$\geqslant$ 0·500

still in excess of that necessary to obtain 99% inhibition in the intact circulation. The main difference here is the time during which the inhibitor acted. We believe these last two experiments demonstrate that the Bohr effect on the initial deoxygenation rate of red cells can be completely

blocked when the inhibitor is given in adequate concentration and for sufficient time to act completely. Figure 5 is the graphic plot of Expt. 14. Only the curve of the final acetazolamide concentration is shown. It lies almost exactly on the deoxygenation curve for the first 70 msec. Since the

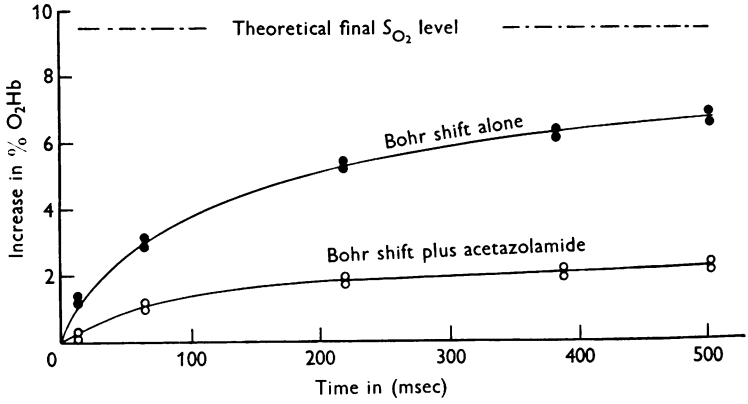

Fig. 6. Time course of Bohr shift alone and acetazolamide-inhibited Bohr shift. Two runs are shown on each curve. Expt. 17. Subject, H. N.

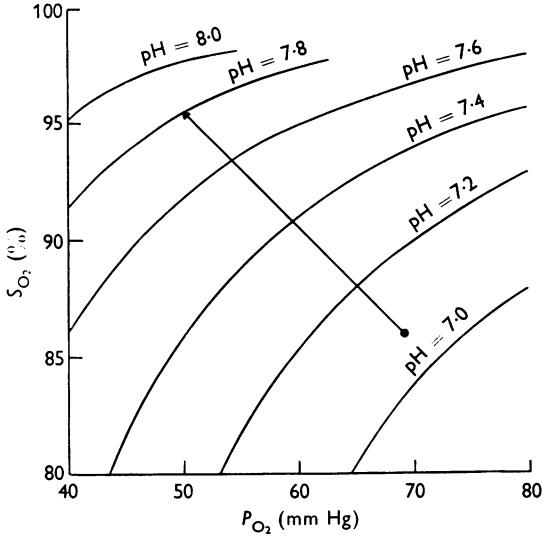

Fig. 7. Initial and final points for the Bohr shift experiment presented in Fig. 6.

equilibrium saturation is the same for the Bohr shift with or without the inhibitor, it must mean that the inhibitor experiment occurs in two steps; the second phase being very slow and not recorded by our short-time experiment.

The results of the experiments of series I–III seemed out of line with the published data on the rate of the Bohr shift. It was important for us to determine the rate of the true Bohr shift in our laboratory. The data for three experiments on three subjects are listed in the first part of Table 3 (series IV) as initial rate (% O_2Hb change per second) and as t_{50}. The initial rates and t_{50} are slightly less than those reported by Craw *et al.* (1963). Since we shifted the pH upwards and they shifted the pH downwards, it appears that the rate of the Bohr shift is about the same in both directions. The upper solid line in Fig. 6 illustrates the results of one of these experiments. Figure 7 shows the shift in the O_2Hb equilibrium curve in this experiment. The basic curve at pH 7·4 is from Bock *et al.* (1924). The other curves for various pH values are calculated using the nomogram of Severinghaus (1958). Admittedly the pH shift from 7·07 to 7·80 is large but it was necessary in order to get a sufficient change in P_{O_2} for our electrodes to record.

The addition of acetazolamide in concentrations of about 5 mg/100 ml. greatly slowed the Bohr shift. The data for three experiments on three subjects are given in the second part of Table 3 (series V). The lower line in Fig. 6 shows the result of one of these experiments. Since we waited only 30 min for the inhibitor to act we may not have blocked the reaction completely; however, the Bohr shift does proceed even in the complete absence of carbonic anhydrase so perhaps we should not expect to find complete suppression by acetazolamide.

DISCUSSION

Physiological significance

When we tried to predict from available data the time course of the dissociation of O_2 from human erythrocytes in the presence of CO_2 loading we foresaw a two-phase reaction: an initial rapid phase due mainly to the deoxygenation reaction alone (Fig. 3) followed by a slow phase due to the Bohr shift (Fig. 6). The observed time course (Fig. 4) did not agree with that prediction. The initial speed of the combined reaction was faster than that of either reaction alone; in fact, it was faster than the sum of the separate reactions. In addition, the long tail of the Bohr shift had gone. According to the definition given at the beginning of the paper we were observing synergistic interaction in the over-all reactions of O_2 and CO_2 with red blood cells.

If the smaller Bohr shift that normally occurs in capillary blood is affected in the same way as in the experiment then the synergistic interaction demonstrated may have an important physiological role. In the tissue and pulmonary capillaries the exchange of O_2 and that portion of

CO_2 exchange due to the hydration reaction (over 60 % of the total CO_2 exchange) will occur simultaneously and rapidly.

Assuming transit times at rest in systemic capillaries to be about the same as in the pulmonary circuit, say 0·75 sec, there is adequate time for both the deoxygenation reaction and the slower Bohr shift reaction to be completed separately (Craw *et al.* 1963). However in exercise, when the red cells move more rapidly through the capillaries, there might not be time enough for the two successive reactions to be completed so that the venous P_{O_2} and P_{CO_2} would incorrectly reflect the end-capillary partial pressures (Forster, Craw, Constantine & Morello, 1962). Our demonstration of the synergistic interaction of the O_2 and CO_2 reactions with consequent acceleration of the exchanges makes it very unlikely that this problem would arise except under bizarre (treatment with acetazolamide) or unusually severe conditions.

Defares & Visser (1962), from equilibrium curves, calculated that CO_2 exchange in the lungs could not occur much faster than O_2 exchange. They predicted that the exchange relations between the gases would be different in systemic as opposed to pulmonary capillaries ('hysteresis'). Our findings support their general thesis as to the close linking of O_2 and CO_2 exchange, but by taking account of rates of reaction rather than equilibria we have shown that the bicarbonate portion of CO_2 exchange proceeds simultaneously with the O_2 exchange. Although we did not perform the O_2 uptake and CO_2 release experiment such as occurs in the lung capillaries we would predict a fast simultaneous exchange in that situation also, that is to say, there would be no hysteresis there either.

Mechanism of the synergism

It is not possible from these experiments or any other evidence available at present to describe precisely the underlying molecular mechanism by which the simultaneous loading and unloading of CO_2 and O_2, respectively, in human erythrocytes shows synergistic interaction. The total process includes: the chemical dissociation of O_2 in a highly reversible, stepwise manner from the haemoglobin, physical diffusion of O_2 through the concentrated haemoglobin solution in the cell interior followed by diffusion across the red cell membrane, diffusion of CO_2 across the red cell membrane and through the concentrated haemoglobin solution in the cell interior, the reaction of CO_2 with water catalysed by the enzyme carbonic anhydrase to form carbonic acid which dissociates into H^+ and HCO_3^-, diffusion of HCO_3^- out of the cell in exchange for Cl^-, the reaction of H^+ with haemoglobin in addition to the alteration of internal cell pH, and some direct reaction of CO_2 with haemoglobin to form carbamino compounds. We shall examine the various processes of diffusion and of chemical reaction in

order to make an appraisal of which processes may be involved in the observed phenomenon.

Kinetics. Since it is readily reversible, the 'pure' chemical reaction for the dissociation of O_2 from O_2Hb may be described by the equation

$$\frac{d(O_2Hb)}{dt} = k(O_2Hb) - k'(O_2)(Hb),$$

where k and k' are the apparent rate constants for dissociation and association, respectively. Unfortunately, the apparent rate constants are not independent of the O_2Hb saturation level since the intermediate compound theory (Adair, 1925) includes eight true rate constants whose values are not all the same (Gibson, 1959). In our study, since the initial saturation is high (97–98 %) we are dealing mostly with the fourth pair of the velocity constants, namely k_4 and k_4', and some data as to their magnitude and pH sensitivity are available. Gibson & Roughton (1954), and Gibson, Kreuzer, Meda & Roughton (1955) have calculated k_4 from data on the rate of replacement of O_2 by CO in fully-saturated O_2Hb solutions. If all their kinetic assumptions are correct then the extrapolated value of k_4 for human haemoglobin at 37° C is about 200 sec^{-1} and it is insensitive to pH over the range 7·1–9·1. On the other hand, k_4' is much larger (perhaps 15-fold) and is pH sensitive (Gibson, 1959). If the chemical reaction is directly involved in the interaction with CO_2 observed in the intact cell the effect is more likely to be due to slowing of the association reaction with decreasing pH than to acceleration of the dissociation reaction. We are emphasizing the pH effect of CO_2 (hydration) because the acetazolamide experiments reported here and by Craw *et al.* (1963) effectively rule out any significant direct or uncatalysed action of CO_2 such as physically dissolved CO_2 or carbamino formation on the O_2Hb reaction kinetics under the conditions of the experiments.

Returning to the intact erythrocyte, the over-all initial cellular dissociation rate constant, k_c, for deoxygenation alone averages 5·2 sec^{-1} (Table 1) which is of the same order as the value of 4·4 sec^{-1} calculated from a kinetic curve of Forster *et al.* (1962, their fig. 4) using the same procedure as we used. If dithionite is used in the extracellular fluid to keep the oxygen concentration effectively zero during the main portion of the O_2 dissociation reaction the value of k_c in human red cell suspensions at 37° C is about 20 sec^{-1} (Lawson, Holland & Forster, 1962). The difference in k_c between the two methods could be due to the early influence of the back reaction on the apparent k_c in the experiments without dithionite. In fact, even with dithionite the back reaction probably has some effect because Lawson (1962) showed that the over-all cellular dissociation rate for intact red cells is pH sensitive in the same direction as is the true

association rate constant (k'_4). It is unlikely that dithionite penetrated the red cells and directly altered the reaction kinetics as it may do in haemoglobin solutions (Roughton, 1963). Therefore some of the pH-sensitive association reaction probably was occurring inside the red cell in Lawson's experiments during the relatively long time it takes for the O_2 molecules to dissociate from haemoglobin and diffuse through the concentrated interior of the erythrocyte and its membrane. In systemic capillary O_2 exchange the extracellular P_{O_2} initially falls in the manner of our experiments rather than to zero as in dithionite experiments. Thus the phenomenon of synergistic interaction, if due to alterations in the association velocity constant, would occur in the capillaries just as in our study.

In summary, if the fundamental cause of the synergism is connected with the 'pure' chemical kinetics, the fourth true velocity constant for the O_2Hb association reaction is the one most likely to be involved. However, we are faced with the problem that the over-all rate of deoxygenation alone in human red cells is only about one tenth as rapid as in solution. This implies that even large changes in true kinetics would have only small effects on k_c.

Diffusion. If we accept the comparative rate constants at face value (Roughton, 1963) then O_2 dissociation in red cells must be largely diffusion-limited and the synergistic interaction that we have observed could be due to alteration in the physical diffusion processes in the cell. At least three possibilities warrant mention.

Roughton (1959) summarized the theoretical and experimental evidence for the theory that nearly half of the diffusion resistance to O_2 passage across the red cell is in the membrane. Kreuzer & Yahr (1960) could not demonstrate that the membrane offers any appreciable resistance, but Sirs (1963*a*, *b*) offered data to support Roughton's view. It is possible that in our experiments CO_2 acted on the membrane to alter O_2 permeability just as trace amounts of metal ions seem to do (Sirs, 1963*a*). There is a difficulty, however. The CO_2 effect can be completely blocked by adequate inhibition of carbonic anhydrase which rules out a direct effect of the CO_2 molecule. The alternative is that the Cl^-–HCO_3^- exchange changes the membrane permeability to O_2. Unfortunately, this begs the question because the data in this paper require that the Cl^-–HCO_3^- exchange process itself, which investigators have shown to be slow (Dirken & Mook, 1931; Roughton & Rupp, 1958), be accelerated by some effect of the O_2 dissociation reaction.

A second possibility is that CO_2 alters the diffusion of O_2 in the concentrated haemoglobin of the cell interior. Again, it cannot be a direct action of the CO_2 molecule because carbonic anhydrase inhibition blocks the

effect. It may be that the molecular re-orientation of haemoglobin that occurs upon removal of oxygen (Perutz, 1963) or the molecular re-arrangement of haemoglobin due to the Bohr shift (Benesch, Ranney, Benesch & Smith, 1961) causes enough stirring within the cell to speed up the Cl^-–HCO_3^- exchange which seems to be the rate-limiting factor in the Bohr shift. The importance of this event, even if it could be proved to exist, depends on the relative resistance of the membrane and the cell interior to diffusion.

A third possibility lies in the application of facilitated O_2 diffusion to the intact red cell. Scholander (1960) presented evidence that facilitation may occur in intact erythrocytes, but he clearly showed that it should not be a factor at the P_{O_2} that normally exist in the body's capillaries. Moll (1962), however, believes that it is important physiologically at oxyhaemoglobin saturations of 75 % or less such as do occur in the course of capillary O_2 exchange in some organs. Roughton (1963) mentions that the original data on intact cells may be incorrect owing to small amounts of haemolysis. If the mechanism did exist it is possible that the pH shifts induced by CO_2 hydration would favour such a transport system. But at the intracellular P_{O_2} and saturation levels used in our experiments this process would cease to be significant.

In summary, the evidence is compatible with the hypothesis that the diffusion process for O_2 or CO_2 or, more likely, the Cl^-–HCO_3^- exchange process that accompanies the Bohr shift may be the primary factors affected when simultaneous loading of CO_2 accelerates the rate of deoxygenation in red cells.

Considering the multitude of events in the complicated process of O_2 and CO_2 reactions with erythrocytes it is likely that more than one factor contributes to the mechanism of synergistic interaction. This should not obscure the phenomenological observation that the simultaneous reactions of O_2 and CO_2 with human red cells are speeded up and may be physiologically advantageous.

SUMMARY

1. Published studies on the rate of the Bohr shift (when the P_{CO_2} of blood is suddenly changed) have shown that the reaction is slower than the deoxygenation reaction alone and could be a factor in limiting capillary oxygen exchange in conditions where capillary blood flow rate is increased.

2. We used a steady-flow, rapid-reaction apparatus to determine the separate and combined rates of deoxygenation and Bohr shift in dilute human red cell suspensions.

3. Our findings on the separate reactions confirm the results of earlier workers, but when the combined reaction rate was measured we found an

unexpected synergistic relation, that is, the combined reaction rate was faster than the sum of the two separate reaction rates.

4. Functionally, this suggests that O_2 and CO_2 exchange reactions are mutually accelerated sc that equilibrium is achieved in tissue and pulmonary capillaries in the time available under almost all conditions.

5. Although we do not know the exact molecular mechanism the evidence available favours a speeding up in the diffusion process of one or more of the component molecules in the reaction rather than a 'pure' chemical kinetic effect.

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